TOYA108.013APC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: HIGUCHI et al.	
	Art Unit: 1623
Application No.: 10/572,404	Examiner: BLAND, LAYLA D
Filing Date: March 16, 2006	
	Attorney DOCKET No.: TOYA108.013APC
For: DRUG AND FOOD OR DRINK FOR IMPROVING HYPERGLYCEMIA	

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O.Box 1450
Alexandria, VA 22313-1450
Dear Sir:

I declare as follows:

1. I am employee of Morinaga Milk Industry Co., Ltd. located at 33-1, Shiba 5-chome, Minato-ku, Tokyo, Japan, which is engaged in the business of production and sale of milk, and other foods.

2. I am one of co-inventors of the above-identified patent application.

3. Ajabnoor describes a "bitter principle" isolated from a drained liquid from the cut leaves of Aloe, and its hypoglycemic activity. However, the drained liquid, which has been regarded as an origin of the bitter principle, contains substantially no 9,19-cyclolanostan-3-ol and 24-methylene-9,19-cyclolanostan-3-ol.

4. I have conducted the experiments described herein and present them as evidence supporting the above fact.

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5. Purpose of the experiments

Measuring amounts of 9,19-cyclolanostan-3-ol and 24-methylene-9,19-cyclolanostan-3-ol in the drained liquid from the cut leaves of Aloe described in Ajabnoor.

6. Method and result of the experiments

Concentrations of 9,19-cyclolanostan-3-ol and 24-methylene-9,19-cyclolanostan-3-ol in the liquid were measured using LC/MS/MS.

(1) Preparation of samples

9,19-Cyclolanostan-3-ol and 24-methylene-9,19-cyclolanostan-3-ol manufactured by the method described in Production Example 1 of WO2006/035525 were designated Standard Compounds.

According to disclosure of Ajabnoor, the bitter principle can be isolated from drained liquid from the cut leaves of Aloe (dried brownish mass, very bitter in taste) (see lines 2-5 in Introduction on page 215). And there is described that this study was undertaken in order to asses hypoglycemic activity which had been reported by Ghannam et al. (see lines 1-5 on page 216).

Therefore, in the present experiment, a drained liquid from the cut leaves of Aloe, which has been regarded as an origin of the bitter principle, was designated as Sample 1.

(2) Preparation of standard solution and internal standard solution

To 1mg of Standard Compounds was added methanol/chloroform(4:1, v/v) respectively, and the compounds were dissolved to prepare standard solution(1mg/ml). Subsequently, methanol/chloroform(4:1, v/v) was added to precision-measured 1mg of Brassicasterol, and Brassicasterol was dissolved to prepare an internal standard stock solution(1mg/ml). The internal standard stock solution was diluted by methanol to prepare an internal standard specimen(10μg/ml).

(3) Method for experiments

[pretreatment of samples]

Pretreatment of samples was performed as described below.

a) To 5ml of each of the samples was added 100µl of methanol (corresponding to the standard solution used for a standard sample solution for creation of a calibration curve), and further added the internal standard solution to obtain mixture.

b) To the mixture was added 5ml of ethyl acetate, and mixed well using a shaking apparatus for 5 minutes.

c) After centrifugation (3,000rpm, 4°C, 2min), organic layer was taken into a glass test tube, and dried in nitrogen gas stream at 40°C.

d) To an aqueous layer after removing the organic layer in c), was added 5ml of ethyl acetate, and mixed well using the shaking apparatus for 5 minutes.

e) After centrifugation (3,000rpm, 4°C, 2min), an organic layer was taken into the glass test tube (containing the dried organic layer obtained in c), and dried in nitrogen gas stream at 40°C.

f) The dried organic layer in the glass test tube was re-fused to 100µl of methanol and injected into LC/MS/MS for measurement.

(4) Method of measurement

[Conditions of HPLC]

HPLC 1100 Series (Agilent Technologies, Inc.)

Column Daisopak SP-100-3-ODS-P, 2.0 mm i.d.×150 mm (DAISO CO., Ltd.)

Mobile phase A: 0.1% acetate B: acetonitrile

(A : B = 20 : 80 → 0 : 100 → 0 : 100 → 20 : 80 → 20 : 80, v/v)

(0.00 → 40.00 → 55.00 → 55.10 → 60.00 min)

Flow rate 0.35 mL/min

Column temperature 40°C

Sample temperature 5°C

Injection volume 10 mL

Run time 60 min

[Condition of MS/MS]

MS/MS API 4000 (AB/MDS SCIEX)

Ionization method Atmospheric pressure chemical ionization

Ion polarity mode Positive

Scan mode Multiple reaction monitoring

Turbo probe temperature 350°C

Interface heater On

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Needle Current 3 μA
Curtain gas flow 10 psi (N_2)
Nebulizer gas flow (gas 1) 40 psi (Air)
Collision gas 5 units (N_2)
Duration time 60 min
Switching valve 0.0-5.0 min, To waste; 5.0-60.0 min, To ion source

Concentrations of the compounds in the samples were calculated by internal standard calibration curve method using Analyst ver. 1.3.1 (AB/MDS SCIEX). A weighting of calibration curve 1/x2 was applied. The concentration of each compound was calculated to three places of decimals.

(5) Standard for selecting of data

[calibration curve]

Based on the result of measurement of the standard sample solution for creation of a calibration curve, a calibration curve was created in the range of concentrations retaining sensitivity and linearity. Evaluation was performed by calculating an accuracy of inverse regression concentration(%) at each of the concentration points.

The accuracy of inverse regression concentration(%) was calculated by the following expression using Analyst ver. 1.3.1 (AB/MDS SCIEX).

Accuracy(%) = 100×inverse regression concentration / preparation concentration (calculated to one place of decimals)

[Criteria]

* Accuracy: 80.0-120.0%

* more than three-fourth points including maximum and minimum points satisfy the criteria of the accuracy

(6) Results of evaluation

The results are shown in the following Table 1.

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[Table 1] Concentrations of 9,19-cyclolanostan-3-ol and 24-methylene-9,19-syclolanostan-3-ol in Sample 1

	Concentration [% by mass]
9,19-cyclolanostan-3-ol	n.d.*
24-methylene-9,19-cyclolanostan-3-ol	n.d.*

n.d.* : Below the detection limit. Note that the detection limit is 0.000002% by mass (20ng/ml).

As shown in Table 1, the concentrations of 9,19-cyclolanostan-3-ol and 24-methylene-9,19-syclolanostan-3-ol in Sample 1 are clearly much less than 0.001% by mass.

7. I further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Miyuki Tanaka
Miyuki Tanaka

Date: April 20, 2009